REMARKS

In this Amendment, claim 1 is amended and claims 2-14 are canceled. Therefore, after entry of this Amendment, claim 1 will be the only claim pending in the Application.

Claim 1 has been amended to recite that the congenic rat exhibits a prolonged time spent in open arms in an elevated plus-maze test compared to said wild-type rat, in place of the limitation "anti-anxiety behavior." This amendment is supported by the specification at, for example, page 49, lines 33-34.

Additionally, claim 1 has been amended to recite that the mutant GPR10 gene contains a G to A substitution at the third position of the coding region. This amendment is supported by the specification at, for example, page 46, line 21-26.

No new matter has been added.

Entry of the Amendment is respectfully requested.

Response to Restriction Requirement

On page 7 of the Office Action, the Examiner requests that Applicants affirm the election of Group I, claims 1-6 and 10-11.

Accordingly, Applicants hereby affirm the election of the invention of Group I, claims 1-6 and 10-11, without traverse, and hereby cancel non-elected Claims 7-9 without prejudice to pursue the same in a Divisional Application(s).

Response to Rejection Under 35 U.S.C. §101

On pages 10-17 of the Office Action, the Examiner rejects Claims 1-6, 10 and 12 under 35 U.S.C. §101, as lacking a credible, specific and substantial asserted utility or well-established utility.

More particularly, the Examiner notes that the specification asserts, *inter alia*, that the claimed congenic rat can be used as a model for treating psychiatric diseases, such as depression or anxiety. However, the Examiner believes that this asserted utility is not credible because human depression has not been linked to mutations in GPR10.

The Examiner contends that mutations in GPR10 are associated with: blood pressure and not obesity in humans; increased body weight, body fat and levels of leptin and insulin and decreased glucose tolerance in mice; and increased mobilization and anti-anxiety like condition in rats. The Examiner concludes that the claimed congenic rat cannot be used as a model for human depression or anti-anxiety because the observed phenotypes vary among species (humans, rats and mice), and therefore the effects of GPR10 mutations in rats are not representative of the effects of GPR10 mutations in humans.

Applicants respectfully traverse the Examiner's rejection, because the present invention has a credible and substantial real-world utility.

As set forth in the MPEP §2107, the credibility of an asserted utility is assessed from the perspective of one of ordinary skill in the art in view of the disclosure and any other evidence of record that is probative of Applicants' assertion. "Clearly, Office personnel should not begin an evaluation of utility by assuming that an asserted utility is likely false, based on the technical field of the invention or for other general reasons." MPEP §2107.02(III)(A).

In the present case, Applicants assert that the congenic rat harboring a mutation in the GPR10 gene can be used as a model for, *inter alia*, treating psychiatric diseases, such as depression or anxiety. From the perspective of one of skill in the art, this is a credible utility for the following reasons.

First, the Examiner's utility rejection is largely based on Bhattacharyya, *Diabetes* 52: 1296-1299 (2003). The Examiner contends that, since this reference teaches an association between GPR10 and blood pressure in humans, an association of GPR10 with depression or anxiety is not credible.

Certainly genes may be linked to more than one phenotype, as even suggested by Bhattacharyya at page 1296, right column:

> Both neuroanatomical studies of the ligand and its receptor, as well as the physiological responses to central administration of PrRP, suggest roles in <u>other processes</u>....

Further, Bhattacharyya et al. actually base their initial hypothesis on data from rat experiments, showing that the rat system was regarded by Bhattacharyya as, and in fact is, representative of the human.

> A role for PrRP in the central control of blood pressure is suggested by the finding of a highly significant elevation in mean arterial blood pressure observed in conscious, unrestrained male rats...." See, Bhattacharyya, page 1296, right column.

Still further, the reason Bhattacharyya found an association between GPR10 and blood pressure in particular, and not some other phenotype, is because Bhattacharyya tested for blood

pressure in a relevant subject population. That is, if one does not test for a particular phenotype, one won't find it.

For at least these reasons, Bhattacharyya simply does not support the Examiner's rejection that the asserted utility is not credible, and in fact, Bhattacharyya lends further support to the asserted utility.

Second, Applicants submit WO 03/080099 as evidence that the present asserted utility is credible (herein referred to as WO'99), a copy of which is attached to this Amendment. As evident from of WO'99, human GPR10 is believed to be associated with diseases of the central nervous system in view of its tissue expression profile (page 55), including "manic excitement" and "depression" at page 72. Further, WO'99 contemplates making non-human transgenic animals to elucidate physiological and behavioral roles of GPR10 (page 102), showing that animal models are considered useful in the context of GPR10. Still further, WO'99 provides that such animals include "rat" (page 8). Of course, WO'99 does not teach or suggest the presently claimed congenic rat with a G to A substitution at the third position of the coding region of GPR10.

Additionally, Applicants submit the following literature:

(1) Maruyama *et al.*, Prolactin-Releasing Peptide as a Novel Stress Mediator in the Central Nervous System, *Endocrinology* 142:5, 2032-2038 (2001).

(2) Lin et al., Prolactin-Releasing Peptide (PrRP) Promotes Awakening and Suppresses Absence Seizure, *Neuroscience* 114:1, 229-238 (2002).

(3) Matsumoto et al., Stimulation of Corticotropin-Releasing Hormone-Mediated adrenocoricotropin Secretion by Central Administration of Prolactin-Releasing Peptide in Rats, *Neuroscience Letters* 285: 234-238 (2000).

These references which relate to the physiology of the GPR10 ligand, PrRP, all employ the rat model. This is not because researchers have a sincere interest in rat or rodent biology, but because one of ordinary skill in the art considers the rat to be a useful model for understanding the physiology surrounding PrRP and its receptor GPR10, as it will ultimately relate to the human system.

In view of the above, one of skill in the art would consider Applicants' asserted utility with respect to the claimed congenic rat to be credible, since the rat is an accepted and established model for studying the physiology of PrRP and GPR10.

Withdrawal of this rejection is respectfully requested.

Response to Rejection Under 35 U.S.C. §112, First Paragraph

In addition, on page 17 of the Office Action, the Examiner rejects Claims 1-6 and 10-11 under 35 U.S.C. § 112, first paragraph, as not being supported by a credible utility.

In view of the Amendments to the claims and the comments above, it is believed that one of skill in the art can practice the invention without undue experimentation.

Particularly, since the rat is an accepted animal model for studying the physiology of GPR10 and its ligand, one of skill in the art is capable of using the presently claimed congenic rat to screen for compounds useful for treating psychiatric diseases, such as depression. It is particularly noted, again, that Bhattacharyya discloses a clear connection between rat and human with respect to PrRP, the ligand of GPR10. See page 1296, right column.

Further, since the amended claims recite a particular mutation which Applicants show exhibits the recited phenotype, the claims are commensurate in scope with the teachings of the specification.

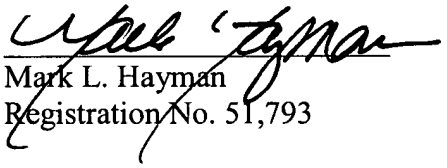
Withdrawal of this rejection is respectfully requested.

Conclusion

In view of the amendments to the claims, and the arguments set forth above, reexamination, reconsideration and allowance of this application are respectfully requested.

The Examiner is invited to contact the undersigned at the below listed number on any questions which might occur.

Respectfully submitted,



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Prolactin-Releasing Peptide as a Novel Stress Mediator in the Central Nervous System*

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ABSTRACT

A1/A2 noradrenergic neurons in the medulla oblongata are well known to mediate stress signals in the central nervous system. Stress activates A1/A2 noradrenergic neurons, and then noradrenaline (NA) stimulates ACTH secretion through hypothalamic CRH. On the other hand, PRL-releasing peptide (PrRP) was recently isolated and was found to be produced by some A1/A2 neurons and the dorsomedial hypothalamic nucleus. We previously demonstrated that PrRP neurons make synapse-like contact with hypothalamic CRH neurons. In fact, we demonstrated that the central administration of PrRP stimulates CRH-mediated ACTH secretion. Furthermore, it has been reported that PrRP neurons in A1/A2 cell groups are colocalized with tyrosine hydroxylase (TH), which is known as the marker enzyme of catecholaminergic neurons. These data strongly suggest that PrRP is related to stress-responsive signal transduction, and PrRP and NA cooperatively modulate the hypothalamo-pituitary-adrenal axis. We therefore examined the effect of water immersion-restraint stress on c-Fos protein accumulation in PrRP- and TH-immunoreactive neurons. The synergistic effects of PrRP and NA on plasma ACTH elevation were also examined. The results clearly showed that c-Fos protein accumulation dramatically increased in the nuclei of A1/A2 and dorsomedial hypothalamic nucleus PrRP neurons. In addition, it was revealed that c-Fos protein was specifically expressed in the PrRP/TH double positive cells in the A1/A2 cell groups. We also demonstrated that the central administration of PrRP and NA in combination at subactive (noneffective) doses clearly induced plasma ACTH elevation. Here we report that PrRP is a novel and important mediator of the hypothalamo-pituitary-adrenal axis for the stress response. (*Endocrinology* **142:** 2032–2038, 2001)

PRL-RELEASING PEPTIDE (PrRP) was recently isolated as a ligand of an orphan seven-transmembrane domain receptor (hGR3) (1). PrRP is known to stimulate PRL release both *in vitro* (1) and *in vivo* (2, 3). It has been demonstrated that PrRP-producing cells exist in the dorsomedial hypothalamic nucleus (DM) and in the A1 region of the ventrolateral reticular formation and the A2 region of the nucleus of the solitary tract in medulla oblongata (4–11). These PrRP-producing neurons extend their axons to magno- and parvocellular neurosecretory cells in the paraventricular hypothalamic nucleus (PVH) and then make synapse-like contact with these cell bodies (4, 12), in which PrRP receptors are known to exist (10). These morphological data strongly suggest that PrRP plays an important biological role in the neuroendocrine system. In fact, we previously found that intracerebroventricular (icv) administration of PrRP significantly increases plasma oxytocin and vasopressin secretion from magnocellular neurosecretory cells in the PVH (13). In addition, we recently found that central administration of PrRP stimulates ACTH and β-endorphin secretion via CRH from the parvocellular neurosecretory cells in the PVH (12).

On the other hand, noradrenergic neurons that project to the PVH are located in A1/A2 cell groups in the medulla oblongata, and a minor portion are found in the locus coeruleus (A6 cell group). These noradrenergic neurons are well known to mediate stress signals in the central nervous system (CNS) (14–19). In fact, lesions of catecholaminergic cell groups in the brainstem or their ascending fibers block or reduce stress-induced changes in the hypothalamo-hypophyseal system (15, 20, 21). In addition, it is commonly accepted that stress activates A1/A2 noradrenergic neurons (18), and noradrenaline (NA) stimulates ACTH secretion through a hypothalamic CRH (19). Interestingly, it has been reported that PrRP neurons in A1/A2 cell groups are colocalized with NA in A1/A2 cell groups (9–11). These data indicate that PrRP mediates stress signals in the CNS as well as NA. The colocalization of PrRP and NA in the same neurons also suggests their synergistic effects. Therefore, we examined the effect of stress on immediate response gene (c-Fos) accumulation in A1/A2 PrRP neurons. The synergistic effects of PrRP and NA on CRH-mediated ACTH secretion were also analyzed in this study. We report here that stress is a potent activator of PrRP neurons, and that PrRP is an important stress mediator in the CNS.

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Materials and Methods

Animals

Adult Wistar male rats were housed in group cages illuminated from 0800–2000 h (12-h cycle). Room temperature varied from 21–24 C. Tap water and laboratory chow were available *ad libitum*. All procedures

were performed in accordance with institutional guidelines for animal care at Saitama University and Takeda Chemical Industries Co., Ltd.

Immunocytochemistry

Proteins were localized as previously described (4, 12, 13). Briefly, the animals were deeply anesthetized and fixed with 5% acrolein in 0.07 M phosphate buffer (pH 7.4). Frozen sections (40 μm) were prepared from the brains. A mouse monoclonal antibody (P2L-1T) and rabbit polyclonal anti-bovine PrRP (no. 8, provided by Takeda Chemical Industries Co., Ltd.) were used as primary antibodies for PrRP. Mouse monoclonal antityrosine hydroxylase clone TH2 (Sigma, St. Louis, MO) and rabbit polyclonal anti-c-Fos (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used for the detection of tyrosine hydroxylase (TH) and c-Fos, respectively. For fluorescence immunocytochemistry for TH and PrRP (shown in Fig. 1), PrRP (labeled with no. 8) and TH were visualized, respectively, with Alexa488-conjugated goat antimouse IgG (Molecular Probes, Inc., Eugene, OR) in red and Alexa594-conjugated goat antirabbit IgG (Molecular Probes, Inc.) in green. For the double staining of PrRP and c-Fos (shown in Fig. 2), PrRP (labeled with P2L-1T) and c-Fos were visualized, respectively, with diaminobenzidine (DAB) in brown and cobalt-DAB in black after labeling with peroxidase by the avidin-biotin-peroxidase complex method. For the triple staining of PrRP, TH, and c-Fos (shown in Fig. 4), PrRP (labeled with no. 8) and TH were visualized, respectively, with Alexa488-conjugated goat antimouse IgG (Molecular Probes, Inc.) in red and Alexa594-conjugated goat antirabbit IgG (Molecular Probes, Inc.) in green, and c-Fos was labeled with peroxidase by the avidin-biotin-peroxidase complex method and then stained with DAB in brown, which changed to blue under a confocal laser microscope.

For morphometry, a complete series of frontal sections (40 μm each) from the caudal end of the area postrema to 1.4 mm posteriorly were analyzed. All neurons in the lateral half of each nuclear area were counted. To avoid double counting, only neurons with a complete nucleus were counted.

Water immersion-restraint stress

Water immersion-restraint stress was performed as previously described (22). Rats were immobilized in stainless restrainers (Natsume, Tokyo, Japan) with the lower half of their bodies immersed in water

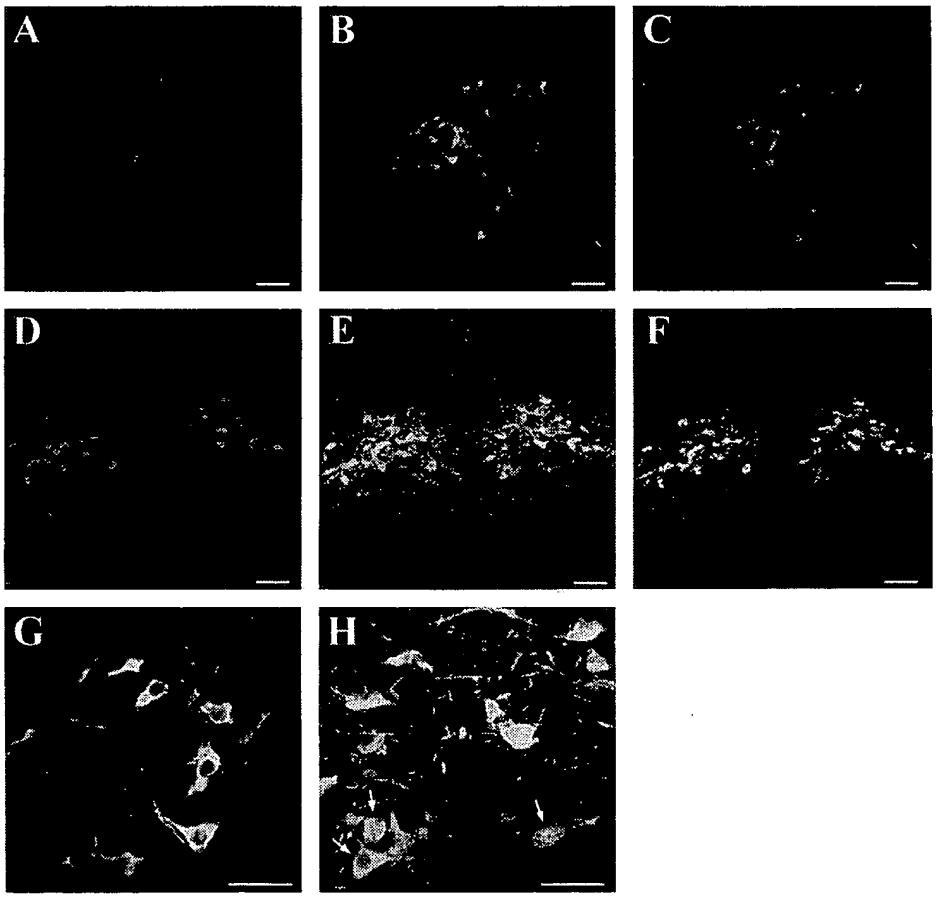

FIG. 1. Photomicrographs showing the double fluorescence immunostaining to locate PrRP and TH in the A1/A2 cell groups. PrRP and TH are stained *red* and *green*, respectively, and the double positive neurons are shown in *yellow*. A–C, Photomicrographs of the A1 region. D–F, Photomicrographs of the A2 region. A and D, Photomicrographs showing only PrRP staining. B and E, Photomicrographs showing only TH staining. C and F, Photomicrographs showing both PrRP and TH staining. G and H, High magnification photomicrographs of C and F, respectively. All PrRP-ir neurons were colocalized with TH in the A1 (A–C and G) and A2 (D–F and H) cell groups. However, TH-ir neurons did not always coexist with PrRP in the A2 cell groups (D–F and H). *Arrows* indicate the PrRP-negative/TH-positive neurons. *Scale bars*, 100 μm (A–F) and 50 μm (G and H).

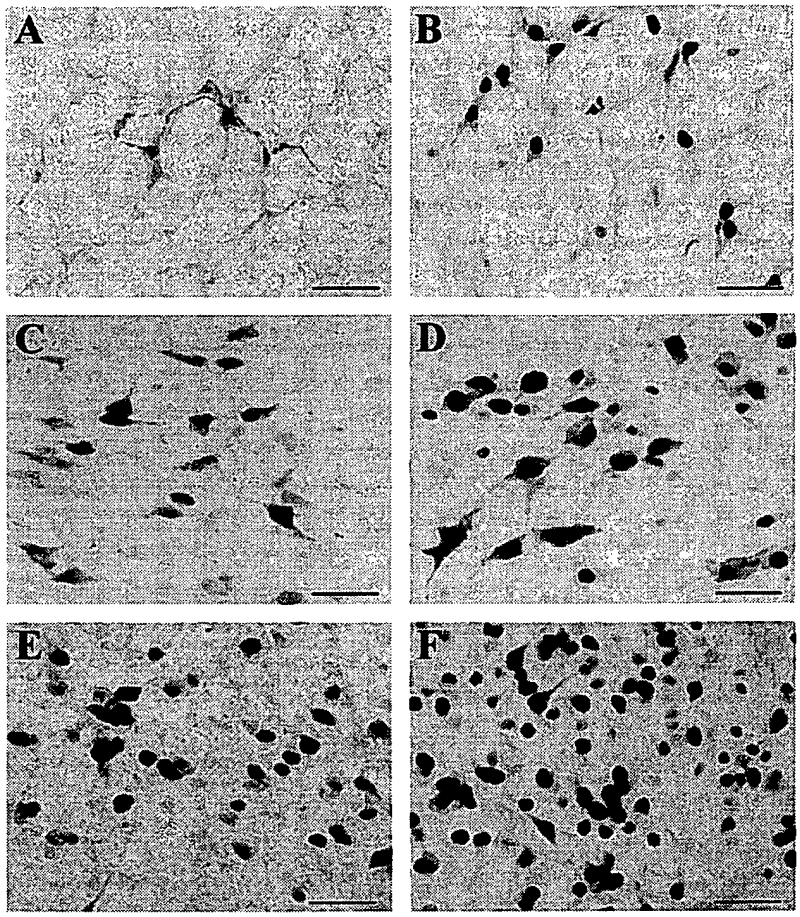

FIG. 2. Effect of water immersion-restraint stress on c-Fos in the PrRP-ir neurons. Photomicrographs show double immunostaining with PrRP and c-Fos antibodies in the A1 (A and B) and A2 (C and D) cell groups, and the DM (E and F). PrRP and c-Fos were stained *brown* and *black*, respectively. In non-stress rats, PrRP-ir neurons expressed some c-Fos protein (A, C, and E). However, when rats were exposed to water immersion-restraint stress for 2 h, c-Fos protein expression dramatically increased in the PrRP-ir neurons (B, D, and F). *Scale bars*, 50 μm.

(25 ± 1 C). They were decapitated 2 or 6 h after the onset of immobilization. Intact (nonstressed) rats were used as a control group.

Synthetic peptide

Rat PrRP31 was synthesized using a combination of recombinant DNA technology and a cysteine-specific cyanylation reaction (23).

Intracerebroventricular administration, blood sampling, and measurement

For icv administration of PrRP, male Wistar rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and then fixed on a stereotaxic apparatus (Narishige, Tokyo, Japan) with the incisor bars adjusted to 3.3 mm below the interaural line. A stainless steel guide cannula (id, 0.4 mm; od, 0.5 mm; AG-8, Eicom, Kyoto, Japan) was inserted into the right lateral ventricle. The stereotaxic coordinates, set according to the atlas of Paxinos and Watson (24), were: anterior-posterior, 7.7 mm above the interaural line; lateral, 1.8 mm from the midline; and height, 6.8 mm above the interaural line. The cannula was fixed to the skull with acrylic dental cement and screws. The guide cannula was occluded with a dummy cannula (od, 0.35 mm; AD-8, Eicom) until the experiments could be performed. The cannula-implanted rats were housed as described above for at least 7 days after the operation. The rats were anesthetized as described above, and then polyethylene tubing (id, 0.5 mm; od, 0.9 mm; SP35, Natsume) for blood sampling was inserted into the right atrium through the jugular vein 1 day before the experiment. PrRP31, NA, or PBS containing 0.5% BSA was injected into the right lateral ventricle. Blood samples were collected via the venous catheter in ice-cooled tubes containing 0.01 M EDTA, 300 kallikrein inhibitor units/ml aprotinin, and 2.5×10^{-4} M *o*-phenanthroline at 15 min pre- and post-icv injection. Plasma was separated from blood and stored at −40 C until the measurement of ACTH. The plasma ACTH concentration was measured with a RIA kit for ACTH (Mitsubishi Yuka Co., Tokyo, Japan). All plasma ACTH measurements were performed between 0900 and 1200 h to avoid the influence of the circadian rhythm. The entire procedure has been described previously (12, 13).

Statistical analysis

The data were analyzed by one-way ANOVA with repeated measurements, and differences between treatment groups were evaluated using Dunnett's multiple test. The statistical significance level was set at $P < 0.05$.

Results

Double immunocytochemistry with PrRP and TH

To determine the relationship between PrRP and A1/A2 NA cell groups, we examined the localization of immunoreactive PrRP and TH in A1/A2 cell groups by fluorescence immunocytochemistry. As previously reported (9–11), the

PrRP-immunoreactive (ir) neurons were colocalized with TH-ir neurons in the A1/A2 cell groups (Fig. 1). In these areas PrRP-ir neurons were always coexpressed with TH-ir neurons (data not shown). Inversely, most TH-ir neurons in A1 cell groups were also positive for PrRP (Fig. 1, A–C and G), but TH-ir neurons in A2 cell groups were not always positive for PrRP (Fig. 1, D–F and H). The results of morphometry are shown in Table 1. In nonstressd rats, the percentages of PrRP-ir cells among the total number of TH-ir cells were 98.4 ± 2.8% and 81.7 ± 3.2% in the A1 and A2 regions, respectively. These values did not significantly change after exposure to water immersion-restraint stress for 2 h in A1/A2 cell groups (Table 1).

Effect of water immersion-restraint stress on the c-Fos expression in PrRP neurons

The immediate response gene c-Fos was used as a marker of neural activation in this study. Rats exposed to nonstress and water immersion-restraint stress for 2 and 6 h were analyzed. Double immunocytochemistry for PrRP and c-Fos clearly showed that water immersion-restraint stress dramatically increased the number of c-Fos-positive nuclei in A1/A2 and DM PrRP-ir neurons (Fig. 2). Morphometry showed that the percentage of c-Fos expression in the PrRP-ir cells of A1/A2 cell groups was significantly increased by water immersion-restraint stress for 2 h compared with that in nonstressed rats, *i.e.* 8.1-fold ($P < 0.01$) in A1 and 3.3-fold ($P < 0.01$) in A2 neurons (Fig. 3). After 6-h stress exposure, it became 8.3-fold ($P < 0.01$) in A1 and 3.5-fold ($P < 0.01$) in A2 neurons. However, the c-Fos activation in DM was weaker than that in A1/A2 cell groups, *i.e.* 1.7-fold ($P < 0.01$) at 2 h and 1.6-fold ($P < 0.01$) at 6 h.

Triple immunocytochemistry for PrRP, TH, and c-Fos in A1/A2 cell groups after exposure to water immersion-restraint stress

Triple immunocytochemistry for PrRP, TH, and c-Fos in A1/A2 cell groups clearly showed that most PrRP/TH double positive neurons also become positive for c-Fos under water immersion-restraint stress compared with those in nonstressed rats (Fig. 4, A and B). However, most PrRP-negative/TH-positive neurons in A2 cell groups were not reactive to c-Fos (Fig. 4, C–F), *i.e.* PrRP-containing NA neurons responded specifically to water immersion-restraint stress in the A1/A2 cell groups. The results of morphometric analysis are shown in Table 2. Morphometry showed that the percentage of c-Fos expression in PrRP/TH-double positive neurons was 17.8 ± 5.0% in nonstressed rats in the A2 region, and that of PrRP-negative/TH-positive neurons was 0.5 ± 0.8%. In the A1 region, the percentage of c-Fos expression in PrRP/TH double positive neurons was 10.8 ± 13.1% in nonstressed rats. On the other hand, the percentage of c-Fos expression PrRP/TH double positive neurons was 90.5 ± 6.2% after exposure to water immersion-restraint stress in the A2 region, whereas that of PrRP-negative/TH-positive neurons was 2.2 ± 2.2%. In the A1 region, the percentage of c-Fos expression in PrRP/TH double positive neurons was 87.9 ± 6.3% after exposure to water immersion-restraint stress. The value for PrRP-negative/TH-positive neurons in the A1 region was not determined because there are almost no PrRP-negative/TH-positive neurons in the A1 region.

TABLE 1. Percentages of PrRP-ir cells among the total numbers of TH-ir cells

	A1	A2
Nonstress	98.4 ± 2.8	81.7 ± 3.2
Stress, 2 h	98.1 ± 1.7	76.2 ± 4.9

In the A2 region, TH-ir cells were divided into two subpopulations, PrRP/TH-double positive neurons and PrRP-negative/TH-positive neurons. The percentage of PrRP-ir neurons among the total number of TH-ir cells is not significantly different between nonstress rats and rats exposed to water immersion-restraint stress for 2 h. Values are the mean ± SEM (n = 3).

Effects of icv administration of PrRP and NA on the plasma ACTH level

The colocalization of NA and PrRP in the A1/A2 cell groups suggests the synergistic actions of these factors on the hypothalamo-pituitary-adrenal axis. We therefore performed coadministration (icv) of subactive (noneffective) doses of PrRP (1 nmol) and NA (0.1 nmol) to examine their synergistic effects on the plasma ACTH level. Blood was collected 15 min pre- and post-icv injection, and the blood collected 15 min before icv injection was used for measurement of basal ACTH levels in individual rats. PrRP (1 nmol) and NA (0.1 nmol) had no effect compared with basal plasma ACTH levels. In contrast, coadministration of PrRP (1 nmol) and NA (0.1 nmol) significantly increased (3.2-fold; $P < 0.01$) the plasma ACTH level compared with basal plasma ACTH levels (Fig. 5). These data suggest that PrRP and NA act synergistically to induce CRH-mediated plasma ACTH elevation.

Discussion

It is generally accepted that A1/A2 NA neurons extend their axons to the PVH and play an important role as stress mediators. These NA stimulate ACTH secretion through hypothalamic CRH (14–21); A1 NA neurons particularly promote vasopressin and oxytocin secretion (25–27). On the other hand, it was recently revealed that PrRP-producing cells exist in the DM in the hypothalamus and the A1/A2 region of the medulla oblongata (4). The axons of PrRP neurons project to the PVH (4), and central administration of PrRP clearly stimulates oxytocin and vasopressin secretion (13) and CRH-mediated ACTH secretion (12). In addition, it has been reported that A1/A2 PrRP neurons as well as the DM are retrogradely labeled after tracer injections in the PVH (11), which indicates that PrRP neurons in A1/A2 regions and also the DM are in direct contact with neurons in the PVH. These locations and the functional coincidence between NA and PrRP neurons strongly suggest that PrRP as well as NA may be related to stress responses in the CNS.

In this study we confirmed previous reports (9–11) showing that PrRP and TH are colocalized in A1/A2 cell groups (Fig. 1). Morphometry showed that PrRP-ir neurons were colocalized, with 81.7 ± 3.2% of the TH-ir neurons in the A2 region and 98.4 ± 2.8% of those in the A1 region (Table 1). Morales *et al.* reported that some TH-ir neurons in the A1/A2

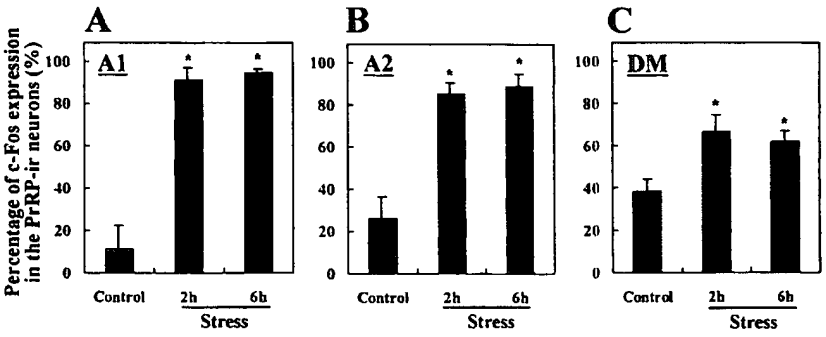

FIG. 3. The percentage of c-Fos expression in PrRP-ir neurons was determined in rats after nonstress (Control) and after exposure to water immersion-restraint stress for 2 and 6 h. The percentage of c-Fos expression in the PrRP-ir neurons was significantly increased in the A1 (A) and A2 (B) cell groups and the DM (C) after exposure to water immersion-restraint stress. Values are the mean ± SEM (n = 3–6). *, $P <$ 0.01 *vs.* control.

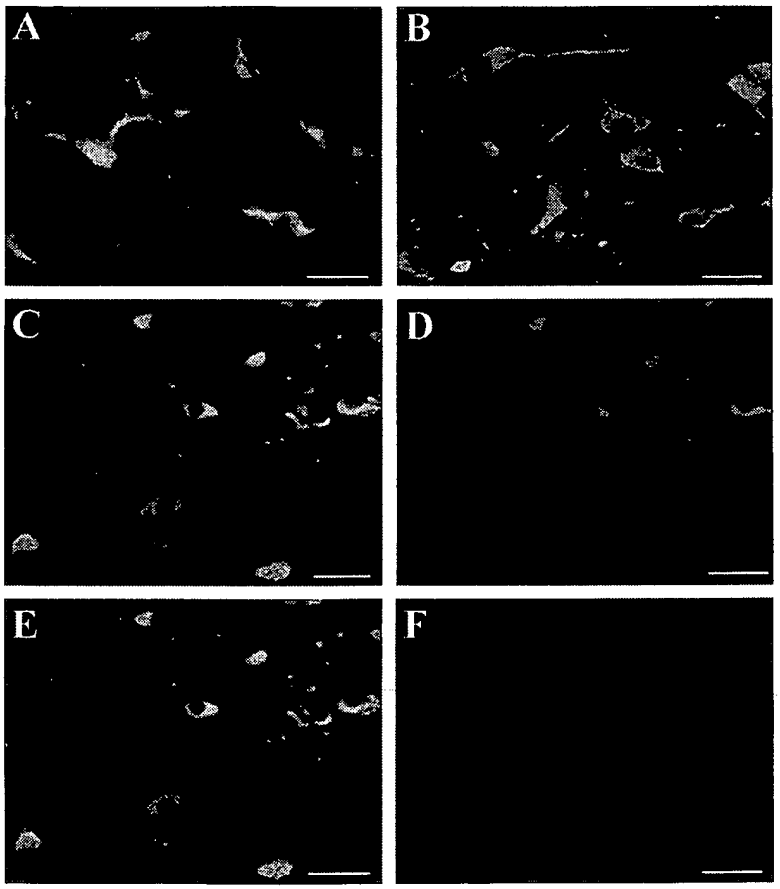

FIG. 4. Photomicrographs showing triple immunostaining with PrRP, TH, and c-Fos antibodies after exposure to water immersion-restraint stress for 2 h in A1/A2 cell groups. PrRP and TH are stained *red* and *green*, respectively, and neurons stained with both are *yellow*. c-Fos is stained *blue*. When rats were exposed to water immersion-restraint stress for 2 h, most PrRP/TH-double positive neurons showed expression of c-Fos protein in the A1 (A) and A2 (B) cell groups. However, TH-ir neurons that showed no PrRP-positive reaction did not always show expression of c-Fos protein in the A2 cell groups (C). D–F, Individual images comprising C, which represent PrRP, TH, and c-Fos, respectively. *Scale bars*, 25 μm (A and B) and 50 μm (C–F).

regions also contain PrRP messenger RNA, *i.e.* 36.6 ± 13.7% of the TH-ir neurons in the A2 region and 35.2 ± 9.4% of those in the A1 cell groups are positive for PrRP messenger RNA (11). Their results were different from our data; however, this may be due to the different detection methods. At least both sets of data show that NA neurons in the A2 cell group are dividable into two subpopulations, *i.e.* PrRP-positive and -negative neurons. To determine whether these PrRP neurons respond to stress, we examined c-Fos expression in PrRP neurons under stress. Our results clearly indicated that water immersion-restraint stress activates A1/A2 PrRP-ir neurons. In addition, triple immunostaining for PrRP, TH, and c-Fos in the A1/A2 cell groups showed that PrRP/TH double positive and PrRP-negative/TH-positive neurons are distinct in the response to water immersion-restraint stress. Morphometry after triple immunostaining for PrRP, TH, and c-Fos also indicated that the percentage of c-Fos expression in PrRP/TH double positive neurons was 90.5 ± 6.2% after

TABLE 2. Percentages of c-Fos protein expression in PrRP/TH-double positive ($PrRP^+/TH^+$) and PrRP-negative/TH-positive ($PrRP^-/TH^+$) neurons

	A1		A2	
	$PrRP^+/TH^+$	$PrRP^-/TH^+$	$PrRP^+/TH^+$	$PrRP^-/TH^+$
Nonstress	10.8 ± 13.1	NE	17.8 ± 5.0	0.5 ± 0.8
Stress, 2 h	87.9 ± 6.3[a]	NE	90.5 ± 6.2[b]	2.2 ± 2.2[c]

In the A1 and A2 regions, c-Fos protein expression was significantly increased in $PrRP^+/TH^+$ neurons after exposure to water immersion-restraint stress. In the A2 region, $PrRP^+/TH^+$ neurons specifically expressed c-Fos protein compared with $PrRP^-/TH^+$ neurons after exposure to water immersion-restraint stress for 2 h. The value for $PrRP^-/TH^+$ neurons in the A1 region was not determined because there were almost no $PrRP^-/TH^+$ neurons in the A1 region. Values are the mean ± SEM (n = 3). NE, Not examined.

[a] $P < 0.01$ *vs.* nonstress (A1, $PrRP^+/TH^+$).
[b] $P < 0.01$ *vs.* nonstress (A2, $PrRP^+/TH^+$).
[c] $P < 0.01$ *vs.* stress, 2 h (A2, $PrRP^+/TH^+$).

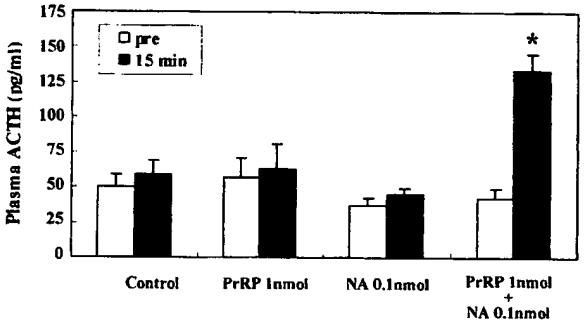

FIG. 5. Effect of coadministration of PrRP and NA into the right lateral ventricle on ACTH release in conscious male rats. The plasma ACTH levels, pretreatment (pre) and 15 min after icv injection (15 min) were compared. The icv administration of 0.5% BSA in PBS (control), 1 nmol PrRP, or 0.1 nmol NA had no effect on ACTH elevation. However, coadministration of 1 nmol PrRP and 0.1 nmol NA induced a large increase in the plasma ACTH level, which suggested that PrRP and NA acted synergistically to induce CRH-mediated plasma ACTH elevation. Values are the mean ± SEM (n = 4). *, $P <$ 0.01 *vs.* pre.

exposure to water immersion-restraint stress in the A2 region, whereas that of PrRP-negative/TH-positive neurons was 2.2 ± 2.2%. These data suggest that a subpopulation of PrRP/TH double positive neurons in the A2 cell group predominantly responded to water immersion-restraint stress.

On the other hand, we previously demonstrated that PrRP (10 nmol) significantly increased plasma ACTH and β-endorphin levels (12). We also reported that this elevation was completely blocked by treatment with α-helical CRH, which clearly indicated that PrRP elevated pituitary ACTH secretion through CRH stimulation. However, we failed to achieve ACTH stimulation with a low concentration of PrRP (1 nmol) (12). To explain the low activity of PrRP on CRH secretion, we examined the colocalization of PrRP and NA in the A1/A2 cell groups. As is well known, the PrRP and NA receptors are localized in the PVH (10, 28), which suggests that PrRP and NA may cooperatively stimulate CRH neurons. Therefore, we examined the effect of coadministration (icv) of PrRP and NA on the plasma ACTH level via the

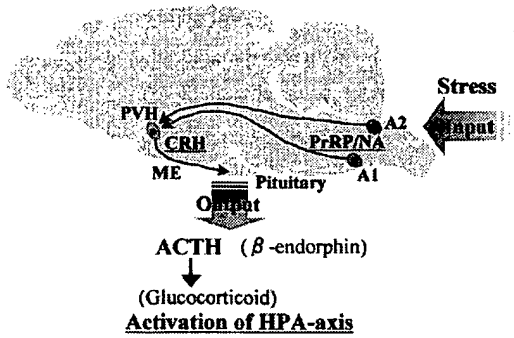

FIG. 6. Schematic representation showing the stress-related function of PrRP. PrRP coexists with medullary A1/A2 NA neurons, and these cells are peculiarly activated by stress. PrRP and NA are discharged from the neural terminals of the PVH and act synergistically to induce CRH-mediated ACTH elevation. ME, Median eminence.

hypothalamic CRH. As a result, we demonstrated that the central administration of PrRP (1 nmol) and NA (0.1 nmol) in combination at subactive doses clearly induced plasma ACTH elevation. This clearly showed that PrRP and NA cooperatively stimulate the hypothalamo-pituitary-adrenal axis.

Some functions of PrRP in the CNS (12, 13, 29–31) have been reported; however, the biological significance of A1/A2 PrRP neurons has not been discussed. In this study we first found that PrRP neurons in the brainstem are related to stress. We also showed that PrRP synergistically acts with NA to induce ACTH secretion. Our data showed that a subpopulation of A1/A2 NA cells expressing PrRP plays a more integral and specific role in stress responses in the CNS compared with neighboring non-PrRP-containing NA neurons. This novel stress-related signal pathway is schematically illustrated in Fig. 6. We believe that our findings suggest the biological significance of PrRP in the CNS. This may be supported by the previous report that icv administration of PrRP increases blood pressure (31) and plasma oxytocin and vasopressin levels (13), which are known to be common stress responses (32, 33). PrRP maybe regulate these stress-relating phenomena as a stress mediator.

The functional differences between PrRP-producing nuclei are not been understood at present. However, it is also known that A1 NA neurons mainly project to the magnocellular division of the PVH and regulate vasopressin and oxytocin neurons, whereas A2 NA neurons mainly project to the parvocellular division of the PVH and regulate CRH neurons (25–27). This indicates the possibility that a functional difference may exist between A1 and A2 PrRP neurons as well as NA. In addition, the c-Fos expression study showed that not only A1/A2 cell groups but also the PrRP-positive cell group in the DM respond to stress stimuli. To explain this phenomenon it is noteworthy that the DM is known to be related to food intake (34), and that PrRP suppresses food intake (30). This suggests that the DM may be related to suppression of food intake during an acute stress response. However, further study is needed to clarify the details of PrRP related-stress signal transduction.

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PROLACTIN-RELEASING PEPTIDE (PrRP) PROMOTES AWAKENING AND SUPPRESSES ABSENCE SEIZURES

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Abstract—Prolactin releasing peptide (PrRP) is a recently identified neuropeptide that stimulates prolactin release from pituitary cells. The presence of its receptor outside the hypothalamic-pituitary axis suggests that it may have other functions. We present here evidence that PrRP can modulate the activity of the reticular thalamic nucleus, a brain region with prominent PrRP receptor expression that is critical for sleep regulation and the formation of non-convulsive absence seizures. Intracerebroventricular injection of PrRP (1–10 nmol) into sleeping animals significantly suppresses sleep oscillations and promotes rapid and prolonged awakening. Higher concentrations of PrRP (10–100 nmol) similarly suppress spike wave discharges seen during absence seizures in genetic absence epilepsy rats from Strasbourg, an animal model for this disorder. In concordance with these findings, PrRP suppressed evoked oscillatory burst activity in reticular thalamic slices *in vitro*.

These results indicate that PrRP modulates reticular thalamic function and that activation of its receptor provides a new target for therapies directed at sleep disorders and absence seizures.

Key words: prolactin releasing peptide, GPCR, sleep, petit-mal seizures, reticular nucleus of the thalamus, thalamic slice.

The natural ligand of the G protein-coupled receptor GPR10 (Marchese et al., 1995; also called hGR3 by Hinuma et al., 1998) and its rat homologue UHR-1 (Welch et al., 1995) was isolated as a 31-amino acid peptide (Hinuma et al., 1998). This peptide was named prolactin releasing peptide (PrRP) because it induced release of prolactin from pituitary cells through activation of the GPR10 receptors. The PrRP receptor is highly expressed not only in the anterior pituitary but also in various hypothalamic nuclei (Hinuma et al., 1998; Roland et al., 1999) and several studies have shown PrRP as an endocrine modulator of hypothalamic hormones such as FSH/LH (Seal et al., 2000), CRH (Matsumoto et al., 2000) and oxytocin (Maruyama et al., 1999b) as well as being involved in the regulation of food intake (Lawrence et al., 2000). Other studies have reported evidence supporting the notion that PrRP functions are not confined to the hypothalamus/pituitary system. Innervation by fiber tracts containing PrRP was found in the amygdala, thalamus, and in the bed nucleus of the stria terminalis (Maruyama et al., 1999a). The receptor for PrRP is present in various brain regions and receptor distribution overlaps in some, but not all, areas with that of the PrRP-positive fiber tracts (Roland et al., 1999; Ibata et al., 2000). These anatomical data suggest that the PrRP system may have broader functions in the brain that are not yet understood.

One brain area with prominent expression of PrRP receptor mRNA is the reticular thalamic nucleus (RTN) (Roland et al., 1999; Ibata et al., 2000), a layer of mainly GABAergic cells that surrounds much of the thalamus like a shell (Jones, 1985). The RTN receives collaterals from thalamocortical and corticothalamic fibers and in return modulates thalamic output through inhibitory projections (Destexhe, 2000; Murray and Guillery, 1996). Because of these interconnections, the RTN is believed to play an important role in controlling the transfer of sensory information to the cortex and also in setting the vigilance state (Steriade et al., 1986). It is of

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Abbreviations: aCSF, artificial cerebrospinal fluid; ECoG, cortical EEG; EDTA, ethylenediaminetetra-acetate; EEG, electroencephalogram; EMG, electromyograph; GAD, glutamate decarboxylase; GAERS, genetic absence epilepsy rats from Strasbourg; HSD, honestly significant difference; PrRP, prolactin releasing peptide; RTN, reticular thalamic nucleus; SWD, spike wave discharges.

particular interest in this regard that thalamic activity can assume two alternative firing modes characterized as tonic firing and as rebound burst firing (Contreras et al., 1993). The latter sustains sleep spindles (7–14 Hz) and occurs during early sleep. The input from the RTN is believed to play a critical role in this form of synchronized activity (Steriade et al., 1985). Characteristic thalamocortical spike-and-wave discharges (SWD) also appear as a pathological marker in the non-convulsive type of epilepsy called absence seizure (petit mal), and abnormalities in the RTN-thalamic circuitry have been implicated in its pathogenesis (McCormick and Bal, 1997; Avanzini et al., 2000).

Given the critical role of the RTN neurons in the generation and control of thalamic oscillatory activity, activation of PrRP receptors in this region could potentially be used to influence sleep/wake states and absence seizures. The present study tested this idea by measuring the effects of PrRP infusion on oscillatory activity in the thalamus/RTN system in an *in vitro* slice preparation and in two *in vivo* paradigms, namely the sleep/wake states in normal rats and the electroencephalogram (EEG) seizures in the genetic absence epilepsy rats from Strasbourg(GAERS).

EXPERIMENTAL PROCEDURES

In situ hybridization and double-labeling in situ hybridization

In situ hybridization was performed as previously described (Winzer-Serhan et al., 1999). Frozen sections (20 μm) were prepared from adult Sprague–Dawley rat brains and fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4). *In situ* hybridization was carried out by incubating the sections overnight at 60°C in hybridization buffer (50% formamide, 10% dextran sulfate, 500 μg/ml tRNA, 10 mM dithiothreitol, 0.3 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) with ^{35}S-labeled GPR10 sense and antisense cRNA probes (10^7 c.p.m./ml). The cRNA probes were prepared from full length GPR10 cDNA (1100 bp) template (gift from Dr. Brian O'Dowd, University of Toronto) in pcDNA3. The template was first linearized with either *Hin*dIII or *Not*I to generate the templates for the antisense or sense cRNA transcripts using SP6 or T7 RNA polymerases, respectively. The cRNA probes were generated by alkaline hydrolysis to approximately 600 bases. The sections were opposed to β-max film with ^{14}C standards of known radioactivity. Some slides were dipped in liquid Kodak NT2B emulsion and exposed for four weeks at 4°C. After development, the sections were counter-stained with Cresyl Violet.

Glutamate decarboxylase (GAD) double-labeling studies were carried out the same way except that hybridization included both ^{35}S-labeled GPR10 cRNA (2×10^7 c.p.m./ml) and digoxigenin-labeled GAD67 cRNA (0.04 μg/ml). GAD67 probe was generated from GAD67 cDNA in pBS (gift of Dr. Edward Jones) using *Bam*H1 and T7 to produce the antisense probe and *Pvu*II and T3 to produce the sense probe. Digoxigenin-UTP was added to the reaction instead of ^{35}S-UTP. All subsequent steps were done according to manufacturer's instructions (Genius kit, Roche).

EEG/electromyograph (EMG) recordings and i.c.v. injections into sleeping animals

EEG and EMG recordings were made from male Sprague–Dawley rats (300–400 g, Sasco, Oregon, WI, USA) according to the method described previously (Berridge and Foote, 1996). Rats were housed in pairs for at least seven days prior to surgery with *ad libitum* access to food and water on an 11/13 h light/dark cycle (lights on 7:00 AM). Two microliters of vehicle or PrRP31 dissolved in vehicle (1 nmol/2 μl; 10 nmol/2 μl) was infused intracerebroventricularly through a pre-implanted cannula (Berridge and Foote, 1996). Artificial extracellular fluid (147 mM NaCl, 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, 2.5 mM KCl, 5.0 mM NaH_2PO_4; pH 7.4) plus 200 μg/ml cytochrome c served as vehicle. Infusions were made over a 2-min period using a microprocessor-controlled infusion pump (Harvard Apparatus, South Natick, MA, USA). Cortical EEG (ECoG) from the frontal cortex and EMG from the dorsal neck muscles were recorded for 60 min prior to infusion and 90 min following infusion. To be scored as a distinct epoch, the appropriate ECoG and EMG activity patterns needed to persist for a minimum of 15 s. Time spent in each state was scored and totaled for the five 30-min epochs of the observation period, two during the pre-infusion period and three during the post-infusion period. The location of the infusion needle was verified morphologically after each experiment. Data were only used for analysis when EEG recordings were electrically adequate and infusion needle placement was accurate. Statistical analysis was performed using a two-way mixed-design analysis of variance with drug treatment as the between-subjects variable and time as the within-subjects variable. Post-hoc analyses were conducted using Tukey's honestly significant difference (HSD) test.

EEG recordings and i.c.v. injection of GAERS

The ECoG was measured with conventional methods (Liu et al., 1992). In brief, six male GAERS (300–400 g) were bilaterally implanted with 4 stainless-steel electrodes at the frontal and parietal cortex, and with a stainless steel guide cannula (AP = −0.8, ML = 1.2, CV = 3 mm, with bregma as reference). Both the guide cannula and EEG electrodes were anchored to the skull using retaining screws and dental acrylic cement. After one week of recovery, a stainless steel injection cannula was introduced into the guide cannula such that it extended 2–4 mm beyond the tip. Five microliters of solution containing 0, 10, 50 or 100 nmol of PrRP31 were injected through the injection cannula over a 1-min period. The EEG was recorded continuously for the duration of the experiment while the animal was freely moving. The rats were carefully watched and were prevented from falling asleep. Seizure duration was determined as the cumulative duration of spindle wave discharge per consecutive 20-min periods (seconds of seizure activity per 20-min recording) before (reference) and at least one hour after the initial injection. Data from six animals receiving the same treatment were pooled. Statistical analysis was performed using the non-parametric Wilcoxon test.

Electrophysiology in thalamic and hippocampal slices

Horizontal thalamic slices (400 μm) were prepared from Sprague–Dawley rats (postnatal 13–15 day) using a vibratome (Leica, VT1000S) (Cox et al., 1997). The slices were transferred to a recording chamber after at least 1 h of recovery and were maintained in superfusion with artificial cerebrospinal fluid (aCSF) equilibrated with 95% O_2/5% CO_2 at 0.5 ml/min. The aCSF contained (in mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 0.63, $NaHCO_3$ 26, glucose 10, bicuculline 0.01, and D-aminophosphonovaleric acid 0.1. Cytochrome c (100 μg/ml) was routinely added to the aCSF to prevent peptide adsorption to tubing; cytochrome c itself had no effect on oscillatory activity in thalamic slices. A glass recording electrode filled with 2 M NaCl was positioned in the RTN and the recording depth was adjusted so as to obtain distinct oscillatory discharges in response to stimulation of the internal capsule. Stimulation (1–15 μA) was delivered with a bipolar nichrome electrode every 20–30 s. Experiments were carried out at 34°C.

Transverse hippocampal slices (400 μm) were prepared from 16–20 days old Sprague–Dawley rats using conventional methods (Arai et al., 1996). Slices were submerged in aCSF containing (in mM): NaCl 124, KCl 3, KH_2PO_4 1.25, $CaCl_2$ 3.4, $MgSO_4$ 2.5, $NaHCO_3$ 26, glucose 10 and cytochrome c

(100 μg/ml). The aCSF was equilibrated with 95% O_2/5% CO_2 and applied at 0.5 ml/min. Extracellular recordings were made from stratum radiatum of the field CA1 in response to activation of Schaffer-commissural fibers in the same stratum. Stimulation was delivered through nichrome bipolar electrodes every 20 s. All experiments were carried out at room temperature.

The data were digitized at 1–5 kHz (thalamic slice) and 5–10 kHz (hippocampal slice) with the Neuronal Activity Acquisition Program (Eclectek Enterprise, CA, USA). Student's paired *t*-test (pre- vs. post-peptide treatment) was employed for statistical analysis.

Drugs

Rat PrRP31 (31-amino acid peptide, synthesized by Research Genetics) was dissolved in distilled water at 1 mM and diluted 100 times in aCSF containing 100 μg/ml cytochrome c (Sigma).

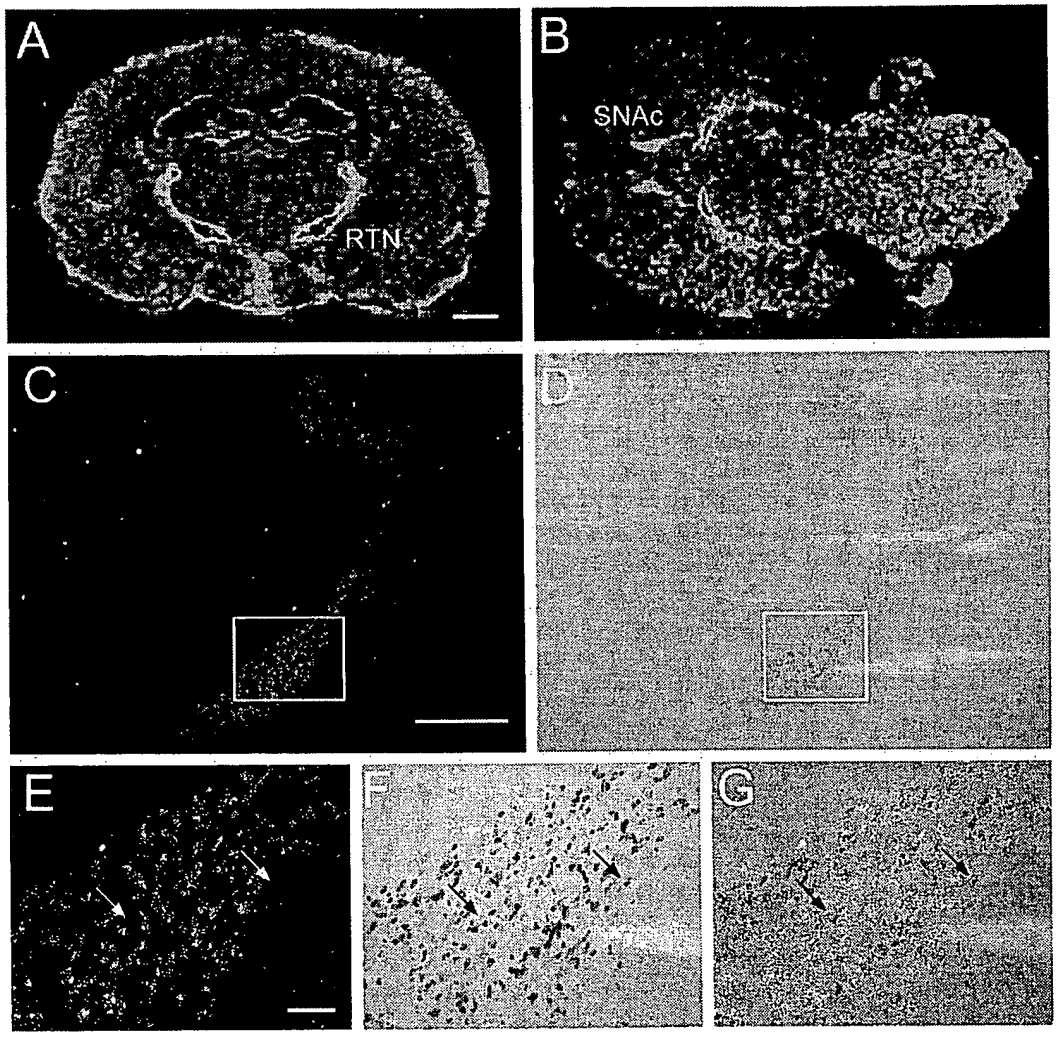

Fig. 1. GPR10 is highly expressed on GABAergic neurons of the RTN. *In situ* hybridization with ^{35}S-labeled antisense GPR10 cRNA on (A) coronal and (B) horizontal sections of adult rat brains. Adjacent sections were labeled with ^{35}S-labeled sense GPR10 cRNA as control; these sections did not show specific staining (data not shown). (C–F) Identification of cell types expressing GPR10 was assessed by double-labeling *in situ* hybridization (see Experimental procedures). (C) Emulsion dipped section visualized under dark field microscopy showing GPR10 labeling within the RTN. (D) The same section exposed to digoxigenin-labeled antisense GAD67 cRNA to identify GABAergic neurons; the section was visualized under light field. (E–F) Higher magnification of (C) and (D) showing that GPR10 is expressed in the same GABAergic neurons. (G) Overlay of emulsion grains and digoxigenin-labeled neurons shows considerable overlap in staining, although some GABAergic neurons are not PrRP receptor positive (arrows). RTN: reticular thalamic nucleus; SNAc: shell, nucleus accumbens. Scale bar = 1 mm, (A–D); 0.1 mm, (E–G).

RESULTS

Expression of GPR10 on GABAergic neurons of the RTN

The PrRP receptor mRNA has previously been shown to be highly expressed in the RTN (Hinuma et al., 1998; Roland et al., 1999). In order to examine if the receptor is expressed in the principal GABAergic cells of this region, a double-labeling *in situ* hybridization experiment was performed using digoxigenin-labeled GAD67 and ^{35}S-labeled PrRP receptor riboprobes. In accordance with the earlier studies, we found that the PrRP receptor is prominently expressed in the RTN (Fig. 1A, B), and its pattern of expression coincided with the 'shell' pattern seen for GAD67 (Fig. 1C, D). Higher magnification showed that PrRP receptor and GAD67 mRNAs are found within the same cells and that nearly all of the PrRP receptor-positive cells are also GAD67 positive (Fig. 1E–G). Some of the GAD-positive cells did not show detectable expression of PrRP receptors (Fig. 1E–G, arrows), but those cells seemed less abundant and it thus appears likely that the PrRP receptor is present on most of the principal GABAergic neurons of the RTN.

Effects of PrRP on sleep/wake states

In a first test whether PrRP can modulate activity in the RTN, we examined its effect on slow wave sleep in animals. PrRP was injected intracerebroventricularly at various doses (1–10 nmol) into sleeping rats. Experiments were carried out during the light cycle when the rats spend the majority of time sleeping. EEG and EMG were recorded for 150 min in three groups, each consisting of six rats, that received either vehicle, 1 nmol PrRP, or 10 nmol PrRP. Analyses were conducted across five consecutive 30-min epochs; vehicle or PrRP were injected at the end of the second epoch. Vehicle-treated rats spent a substantial portion of the entire testing period asleep with characteristic EEG/EMG recording (Berridge and Foote, 1996). Infusions of PrRP elicited dose-dependent increases in the EEG/EMG indices of waking (Fig. 2A); PrRP significantly increased total time spent awake (treatment, $F_{(2,15)} = 24.4$, $P < 0.001$; time, $F_{(4,60)} = 19.5$, $P < 0.001$; treatment × time, $F_{(8,60)} = 8.4$, $P < 0.001$; Fig. 2B) and accordingly decreased total time spent asleep (slow-wave sleep+REM sleep). Post-hoc analyses indicate that total time spent awake was significantly increased during post-infusion epochs in rats treated with 10 nmol PrRP with only a trend at the 1 nmol dose. The increase in awake time at the higher dose was mirrored in the near-complete suppression of slow-wave sleep (treatment, $F_{(2,15)} = 20.7$, $P < 0.001$) (Fig. 2C).

Effects of PrRP on absence seizure formation in GAERS

Absence seizures are characterized by SWD that are similar to the spindle wave oscillations seen during sleep and that originate, like the latter, from the reticular thalamus. In certain genetic rat strains, such as the Wistar strain called GAERS (Danober et al., 1998), such seizure activity occurs spontaneously. Their seizure episodes are characterized by behavioral arrest and recurrent EEG activity exhibiting bilateral synchronous SWD and they are for this reason considered to be an animal model for absence seizures (Marescaux et al., 1992). These animals were used to examine if PrRP is effective in controlling SWD during these seizures. The EEG was recorded throughout the course of the experiment and the time spent in SWDs was cumulated over 20-minute intervals from the initial i.c.v. infusion of either aCSF control or one of three peptide concentrations (10, 50 and 100 nmol). Vehicle-treated rats exhibited recurrent SWD throughout the recording session (Fig. 3A) that did not differ significantly from the activity during the 20-min period preceding the injections (mean cumulated duration of SWD: 340 to 367 s). Injection of PrRP suppressed the EEG seizures as shown in Fig. 3B, C. At the 10-nmol dose, effects were statistically significant but short-lasting; SWD duration was reduced by about 50% during the first 20 min and returned to baseline activity after 40 min. More potent and sustained effects were observed at the higher peptide concentrations. Seizure activity during the first 20 min after injection was reduced by $76 \pm 22\%$ ($n = 6$) at 50 nmol PrRP and by $99 \pm 1.0\%$ ($n = 6$) at 100 nmol PrRP. In both cases, SWD duration was still reduced by more than 50% one hour after the injection.

Effects of PrRP on oscillations in the reticular thalamic slice system

The *in vivo* studies demonstrated that i.c.v. application of PrRP promotes wakefulness in normal animals and suppresses absence seizures in GAERS. These experiments were not designed to address which brain regions are responsible for mediating the peptide's effects, but the well-known role of the RTN in both of these *in vivo* paradigms and the high expression of the PrRP receptor in this region made it seem likely that PrRP directly influenced reticular thalamic activity. To test this proposition, we employed a thalamic slice preparation which has been shown to maintain sufficient reciprocal synaptic connections to generate oscillatory activity (Cox et al., 1997) not unlike the sleep oscillations and the epileptic slow wave discharges. A single stimulation pulse given to the input fibers in the internal capsule triggered repetitive burst firing in the RTN which typically consisted of 6–10 bursts at a frequency of 3–5 Hz (Fig. 4). Application of PrRP (1–20 μM) reduced the number of these oscillatory bursts in a concentration-dependent manner (Fig. 4A–D). The onset of the reduction occurred within 5 min after injection, but washout of the effect was relatively slow and variable. The average reduction at 10 μM was $28 \pm 5\%$ ($P < 0.005$, $n = 12$) and slightly larger effects (33%) were seen in two experiments at 20 μM. At these concentrations PrRP does not cause generalized disturbances in synaptic transmission as shown with slices from the hippocampus, a region in which the PrRP receptor is not expressed. In this prep-

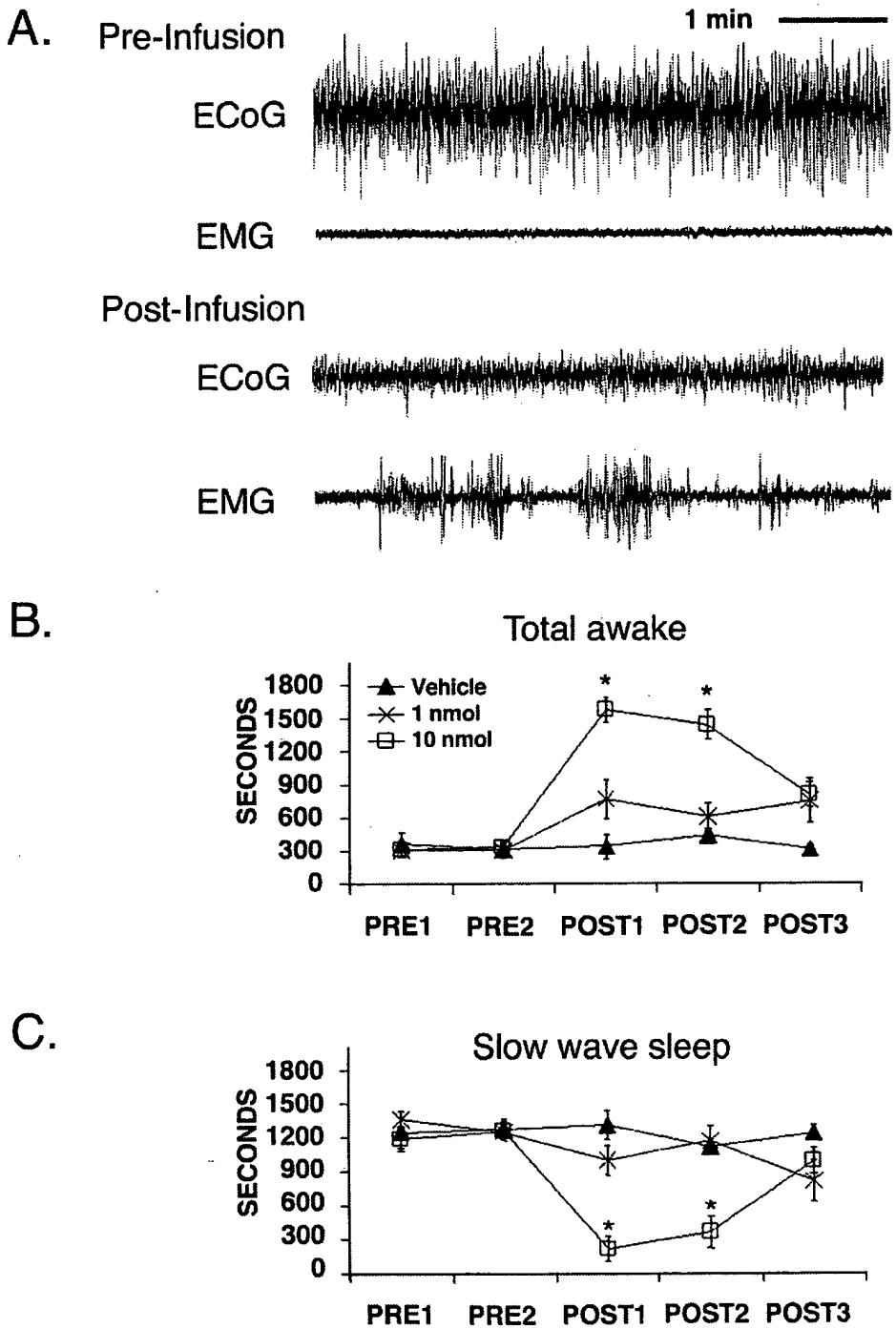

Fig. 2. PrRP induces waking and suppresses slow wave sleep. (A) Representative EEG/EMG traces of pre- and post-injection effects of PrRP (10 nmol) on sleeping animals. (B, C) Effects of varying concentrations of PrRP infused into the lateral ventricle on (B) total time spent awake and (C) slow wave sleep. Symbols represent mean ± S.E.M. (six animals) of the time (s) spent in the two different behavioral state categories per 30-min epoch. PRE1 and PRE2 represent pre-infusion epochs. POST1–POST3 represent post-infusion epochs. Vehicle-treated rats spent a substantial proportion of the testing period asleep. Total time spent awake was significantly increased during the POST1 and POST2 epochs in the 10 nmol group. $^{*}P < 0.01$; $^{+}P < 0.05$ (Tukey's HSD test) compared to vehicle-treated animals.

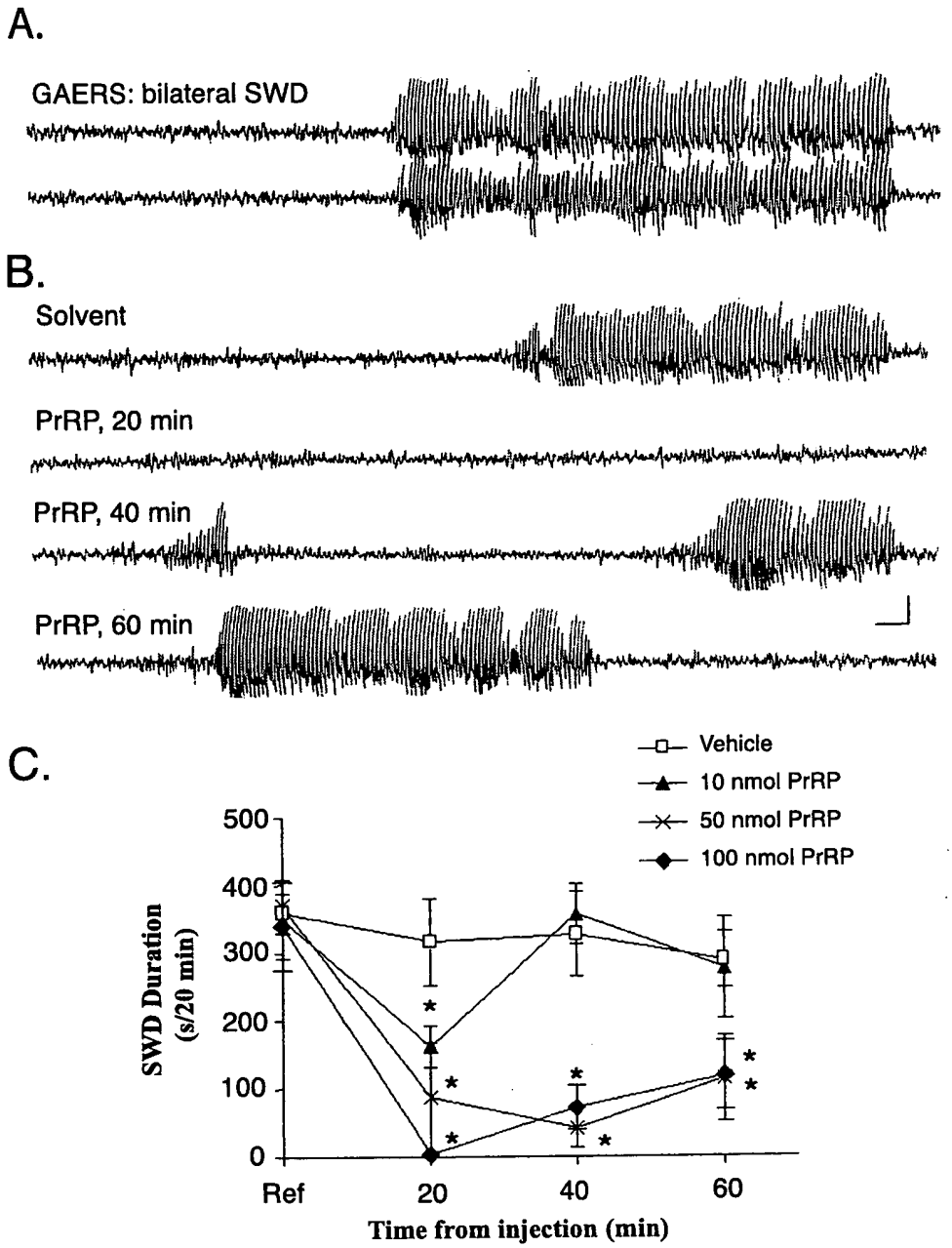

Fig. 3. PrRP suppresses absence seizure activity in GAERS. (A) Representative EEG with SWD in a GAERS: bilateral frontoparietal SWD before injection. (B) Changes in EEG pattern after i.c.v. injection of PrRP (100 nmol). Calibration: 200 μV/1 s. (C) Cumulative duration of SWD per 20 min in GAERS (mean ± S.E.M.) before injection (Ref) and 20, 40, and 60 min after injection of vehicle or 10, 50, or 100 nmol PrRP. $^*P < 0.001$.

aration, PrRP at 10 μM had no detectable effect on amplitude (4.6 ± 5.7%, $n = 6$) or half-width (2.5 ± 1.6%) of synaptic responses (Fig. 4E). Taken together, these results suggest that PrRP specifically suppresses thalamic oscillations and that it does so through the PrRP receptor on the principal RTN neurons.

DISCUSSION

The present study showed that PrRP has an inhibitory action on rhythms generated in the RTN and that it can thereby influence processes such as sleep and absence seizures. These effects are likely to be mediated by recep-

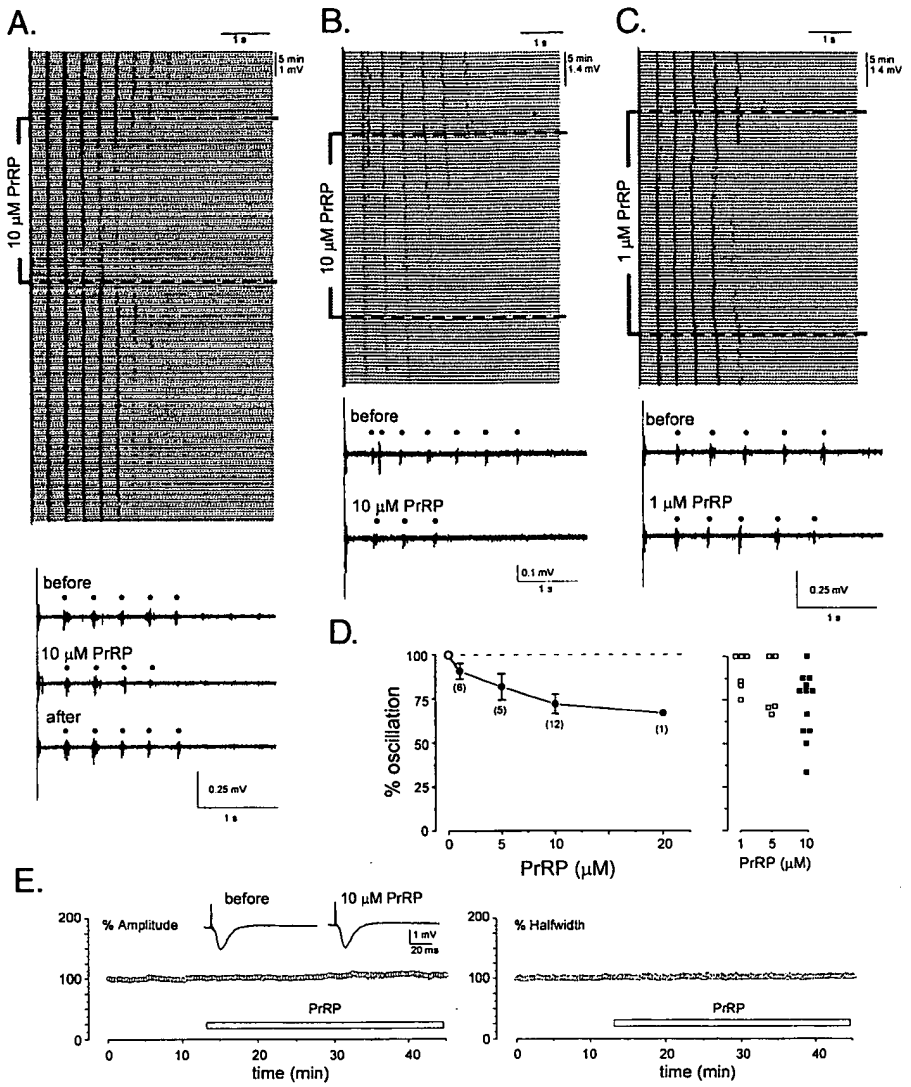

Fig. 4. PrRP suppresses thalamic oscillations *in vitro*. Oscillatory activity was recorded from the RTN in response to stimulation of the internal capsule at 30-s intervals. Each block contains 100 or 195 sweeps lasting 5 s. After establishing a stable baseline for 10–15 min, PrRP was applied to the recording chamber for about 20–25 min as indicated with dashed lines (A–C, top). The traces shown underneath each block were taken immediately before and during PrRP application. The stimulus artifact was truncated. The burst discharges counted for analysis are indicated with dots. (A) Representative experiment with 10 μM PrRP. Note that some recovery was observed upon washout of the peptide. The number of the burst discharges was reduced from six to four (67%) in the presence of PrRP. (B) Another representative experiment with 10 μM PrRP. The burst discharge indicated with a triangle originated from a cell (or cells) adjacent to the recording electrode, which is not participating in lasting slow oscillatory activity (bottom). PrRP reduced the number of bursts from 6 to 3 (50%). (C) Representative experiment with 1 μM PrRP. This concentration of PrRP reduced the intensity of the burst discharge but not the number of bursts. (D) Summary. Concentration–response relations of PrRP's effect on thalamic oscillation. % Oscillation denotes the number of burst discharges immediately before washout of the peptide relative to that of baseline oscillations. The number in parentheses indicates the number of experimental cases. The graph on the right shows the distribution of the PrRP effects at the lower three concentrations, each point representing a single experiment. (E) Effects of PrRP on synaptic transmission in field CA1 of the hippocampus. Transverse hippocampal slices were prepared from young rats (16–20 days) and maintained submerged in aCSF. After establishing a stable baseline, 5–10 μM PrRP was applied as indicated with horizontal bars. The amplitude and the halfwidth of each response was normalized to that of the baseline response and plotted against time. The symbols represent the mean and S.E.M. of six experiments. Representative traces taken immediately before and during application of 10 μM PrRP are shown at the top.

tors in the RTN itself because oscillations in thalamic slices were similarly affected by the peptide. We also confirmed previous findings (Hinuma et al., 1998; Roland et al., 1999) that mRNA for this receptor is prominently expressed in this structure and further clarified with double-labeling *in situ* hybridization that the receptor is present on the principal GABAergic neurons of the RTN. The receptor most likely is located postsynaptically in dendrites and soma of these cells, as binding of radiolabeled PrRP was found in the RTN rather than in the target regions of their axons (Roland et al., 1999).

Effects of i.c.v. injection of PrRP on sleep/wake states

When injected into animals during their sleep phase, PrRP induced a robust change in the EEG/EMG patterns. Slow wave sleep characterized by high amplitude, low frequency waves and a silent EMG was replaced within minutes by normal waking patterns, and the animals exhibited visible behavioral indices of waking. Conversion to the awake state was nearly complete at a dose of 10 nmol and lasted for well over an hour. These findings may seem at variance with a recent study by Zhang et al. (2000, 2001) who reported an increase in REM and non-REM sleep following chronic injection of PrRP. Differences in the experimental design are likely to account for this disparity. The present study examined the animals for 90 min following acute injection of PrRP and the tests were carried out during the light period when animals are normally asleep. Zhang et al. (2000) used chronic injection over 10 h, starting during the dark cycle, and scored behavioral changes over an extended 12-h period. If acute treatment with PrRP promotes awakening as indicated by our observations, the increase in sleep parameters seen in their study may have resulted from a 'rebound' increase in REM or non-REM such as following sleep deprivation (Endo et al., 1997). In molecular terms, chronic infusion of PrRP may have caused progressive desensitization of the receptor and hence an inability of the PrRP system to maintain an adequate vigilance state during the later part of their sessions. More detailed studies concerning the timing of PrRP administration and changes in sleep patterns will be required to resolve this issue.

PrRP directly modulates oscillatory activity in the reticular thalamus in vitro

PrRP suppression of SWD in the GAERS animals exhibited a time course similar to that seen in the sleep/wake experiments. The duration of slow wave discharge was reduced by about 50% at the 10-nmol dose, and injections of at least 50 nmol were needed for effective suppression of seizure activity. This indicates that a higher degree of PrRP receptor occupancy may be needed to suppress seizures than to reduce sleep states. Overall, the concentration range and the time course of the PrRP effect in the *in vivo* experiments were comparable to those in other studies. For instance, Matsumoto et al. (2000) found that 10–20 nmol PrRP i.c.v. transiently increased plasma ACTH levels with a time profile comparable to that reported here and that it induced c-fos expression in the hypothalamus. Nonetheless, the effects observed here most likely were exerted through a direct action on the receptors in the RTN rather than through extrinsic pathways or modulation of ascending modulatory systems. The reason is that oscillatory discharges in thalamic slices, in which activity is disconnected from those external influences, were reduced by PrRP in a manner that strongly resembled the effects on EEG activity in intact animals. The effect in slices was concentration dependent with an approximately 30% reduction in the number of oscillatory bursts at 10–20 μM, a concentration that was estimated to be similar in magnitude to that attained in the brain after i.c.v. injections of 10 nmol peptide. The lack of effect in hippocampal slices appears to rule out the possibility that the concentrations of PrRP employed in the present study disturb excitatory synaptic transmission in a non-selective manner. Taken together, our results suggest that PrRP suppresses oscillatory discharges in the thalamus, presumably through activation of PrRP receptors in the RTN, and that the same effect accounts for the regulation of sleep/wake cycles and for the suppression of absence seizures in GAERS animals. The possibility can of course not be dismissed that effects on other brain structures contributed to the observed *in vivo* actions. However, regions which play a prominent role in sleep-wake regulation such as the preoptic nucleus and the suprachiasmatic nucleus have only moderate to low levels of PrRP receptor expression (Roland et al., 1999; Ibata et al., 2000; unpublished data) and there is no evidence that those areas play a role in the formation of absence seizures. Similarly, PrRP receptor expression is almost undetectable in the locus coeruleus (unpublished data) which is pivotal in regulating arousal. A direct action of PrRP on the RTN thus remains perhaps the most plausible explanation.

Cellular mechanisms for PrRP's action on the reticular thalamic neurons

PrRP most likely modulates the GABAergic output from the RTN that is critical for maintaining thalamic network activity. At the cellular level, PrRP receptor activation has been described to initiate various biochemical events via coupling to G_q, including an increase in intracellular calcium and arachidonic acid release (Hinuma et al., 1998), but the mechanisms whereby PrRP suppresses oscillatory burst activity remains to be investigated. Generation of oscillatory spindle wave discharges is critically dependent on the intrinsic membrane properties of the thalamic cells, and in particular on potassium conductances (I_h, I_K) (Luthi et al., 1998). Activation of various G protein-coupled receptors in the RTN through NE, ACh, and 5HT suppresses a potassium current (Ik), resulting in a depolarization of the membrane potential and the abolition of low threshold calcium spikes that are critical for maintaining oscillatory rhythms (26). Certain locally released neuropeptides such as CCK regulate spindle wave activity in a similar manner (Cox et al., 1997; Lee and McCormick,

1997). Modification of these or similar conductances by PrRP is thus a likely possibility.

Does the PrRP system have a physiological role?

Although exogenously applied PrRP evidently is able to modulate thalamic activity, the question remains if such activity is regulated physiologically by endogenously released peptide. Immuno-histochemical studies so far have failed to detect PrRP fiber terminals in the vicinity of the RTN, except for a high density in the mediodorsal thalamic nucleus, a region which, interestingly, does not show receptor expression (Maruyama et al., 1999a; Ibata et al., 2000). Two discrete nuclei, the nucleus tractus solitarius in the brainstem and the dorsomedial hypothalamus, contain PrRP mRNA and immunoreactivity (Lee et al., 2000; Chen et al., 1999; Maruyama et al., 1999a) but there are currently no indications from the literature of retrograde or anterograde tract tracing studies to demonstrate connections from either of these regions to the RTN (Morales et al., 2000). An alternatively possibility would be that PrRP reaches the RTN through the cerebrospinal fluid after being released elsewhere, for instance from the abundant PrRP immunoreactive fibers seen near the ependymal cells of the third and lateral ventricles (Iijima et al., 1999). Future studies will have to determine if any of these sources release PrRP in concentrations sufficient to reach and activate the receptors in the RTN.

CONCLUSIONS

We have shown that PrRP is capable of modulating thalamic network activity. Although the physiological significance of this system remains to be determined, targeting the receptor of PrRP may provide a new avenue for the management of absence seizures, and possibly, for sleep disorders. Recent efforts to find new pharmacological approaches have focused on $GABA_B$ receptor and NMDA receptor antagonists, both of which inhibited EEG seizure in GAERS animals and evoked oscillations in thalamic slices (Liu et al., 1992; von Krosigk et al., 1993; Peeters et al., 1989). However, these receptors are widely distributed throughout the brain and blocking them is likely to result in general disturbance of synaptic plasticity (Collingridge and Singer, 1990; Davies et al., 1991) and cognitive function. In contrast, PrRP receptors because of their restricted distribution in the brain may allow for a much more focused intervention.

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Stimulation of corticotropin-releasing hormone-mediated adrenocorticotropin secretion by central administration of prolactin-releasing peptide in rats

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Abstract

Prolactin-releasing peptide (PrRP) is a recently isolated hypothalamic peptide which is an endogenous ligand to an orphan receptor. We previously demonstrated that PrRP neurons are widely distributed throughout the rat brain and suggested that PrRP may have important functions in the central nervous system. To analyze the function of PrRP, we studied the effect of intracerebroventricular (i.c.v.) PrRP administration on c-Fos protein accumulation in the rat brain. The results clearly indicated that c-Fos protein accumulation was dramatically increased in the nuclei of corticotropin-releasing hormone (CRH)-positive parvocellular neurosecretory cells in the paraventricular nucleus (PVN). We also demonstrated synapse-like contact between PrRP neurons and CRH cell bodies in the PVN, which suggests that PrRP31 has some effect on CRH secretion. We therefore investigated the effect of i.c.v. administration of PrRP31 on the CRH-mediated increase in adrenocorticotropin (ACTH) levels, and found that plasma ACTH levels were indeed increased by i.c.v. PrRP31. In addition, animals pre-treated with intravenous α-helical CRH, a potent CRH antagonist, showed attenuated plasma ACTH responses after i.c.v. PrRP31 administration. These results strongly suggest that PrRP affects the hypothalamic-pituitary-adrenal axis.

Keywords: Prolactin-releasing peptide; Corticotropin-releasing hormone; Adrenocorticotropin; c-Fos protein; Rat; Immunocytochemistry

We recently isolated a novel bioactive peptide, prolactin-releasing peptide (PrRP), from bovine hypothalamus, and demonstrated it to be a ligand of an orphan seven-transmembrane-domain receptor, hGR3 [3]. PrRP shows specific prolactin (PRL)-releasing activity in vitro [3] and in vivo [10,16]; however, its other biological actions have yet to be fully elucidated.

To investigate the biological significance of PrRP, we previously performed an immunocytochemical study to determine the localization of PrRP neurons in the rat. This revealed that PrRP neurons are widely disseminated throughout the brain, including regions such as the paraventricular nucleus (PVN), the supraoptic nucleus (SON) and the bed nucleus of the stria terminalis (BST) [8]. This distribution pattern suggested that PrRP is involved in a variety of brain functions.

Corticotropin-releasing hormone (CRH)-producing neurosecretory cells are known to be localized mainly among the parvocellular neurosecretory cells of the PVN, which is innervated by PrRP neurons. This led us to suspect that some relationship may exist between PrRP and CRH neurons, and that PrRP neurons may play a role in the functioning of the hypothalamic-pituitary-adrenal (HPA) axis.

The purpose of the present study was to examine the effect of PrRP on CRH regulation in the brains of male rats. First, we performed immunocytochemistry to investigate the effect of intracerebroventricular (i.c.v.) administration of PrRP31 on c-Fos protein (FOS)-related immunoreactivties (-RI) in

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the PVN. Second, we examined the effect of i.c.v. administration of PrRP31 on plasma adrenocorticotropin (ACTH) and β-endorphin levels. We also examined the effects of pretreatment with a CRH receptor antagonist (α-helical CRH) on the increase in plasma ACTH levels stimulated by central administration of PrRP31.

Rat PrRP31 was synthesized using a combination of recombinant DNA technology and a cysteine-specific cyanylation reaction [12].

The animals used in all studies were adult male Wistar rats, weighing 320–350 g at the time of testing. The animals were housed individually in a temperature- (21–24°C) and light- (12 h light–dark cycle, lights on at 08:00 h) controlled room, and had free access to food and water. All study procedures were performed in accordance with institutional guidelines for animal care at Takeda Chemical Industries Ltd. and Saitama University.

Two hours after i.c.v. administration of PrRP31 (20 nmol/rat), the animals were deeply anesthetized and fixed with 5% acrolein. Frozen sections (40 μm) were prepared from the brains and subjected to double immunocytochemistry according to the method described in our previous study [8]. Briefly, the sections were first stained with Fos antibody (Santa Cruz Biotechnology, CA) or CRH antibody (HAC-HM04-01RBP90). After labeling with peroxidase by the ABC method, the sections were stained black with cobalt-diaminobenzidine (DAB) [4]. A second immunocytochemical procedure was then performed using CRH or PrRP [8] antibodies, and the products were labeled with peroxidase by the ABC method. The immunocomplex was stained brown with DAB and examined by light microscopy.

A cannula was inserted into the right lateral ventricle of each rat under anesthesia. The rats were allowed to recover for at least 7 days, and were handled daily before performing the subsequent experiment. A polyethylene tube for blood sampling was inserted into the right atrium through

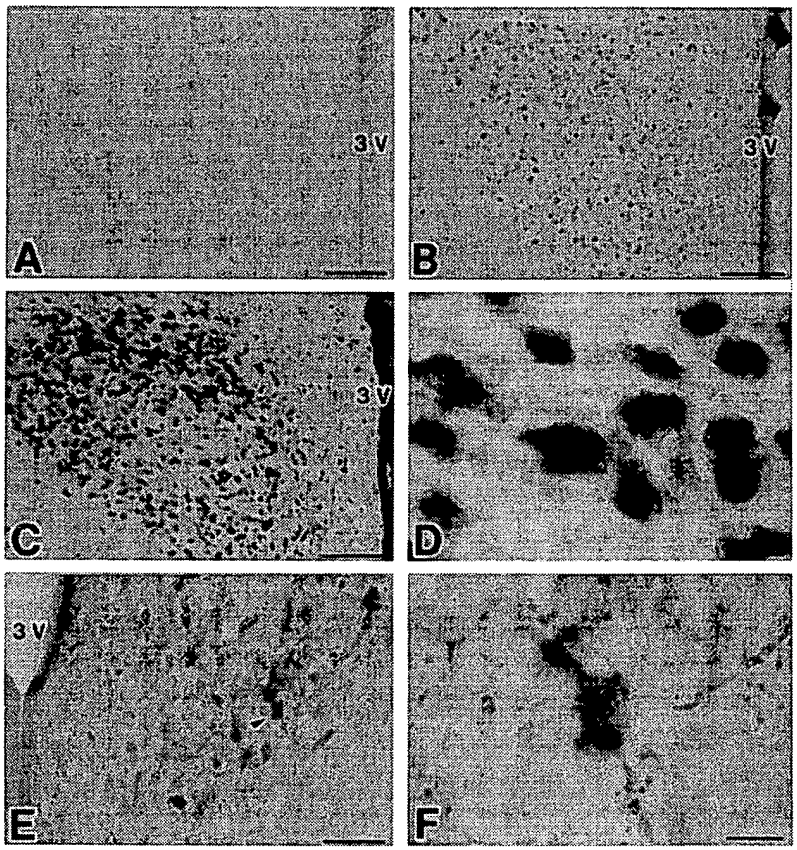

Fig. 1. Photomicrographs showing the changes in FOS-RI, and the double immunostaining technique used to localize FOS/PrRP and CRH, in the PVN after central administration of PrRP31. (A) A section from a control (vehicle-treated) rat. (B) A section from a PrRP31-treated (20 nmol) rat. (C) A section showing the coexistence of FOS-RI (black) and CRH (brown) in the PVN after PrRP31 treatment. (D) An enlargement of the section shown in C. The animals were decapitated 120 min after i.c.v. administration of PrRP31. (E) Synaptic contact (arrows heads) between PrRP fibers (brown) and CRH cell bodies (black). (F) An enlargement of typical cells from the section shown in (E). 3V, third ventricle. Scale bars, 100 μm in (A–C,E); 10 μm in (D,F).

the jugular vein 1 day before the experiment. PrRP31 (1 or 10 nmol/rat) or phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) was injected into the right lateral ventricle. Blood samples were taken from the venous catheter 15 and 0 min before, and 5, 15, 30, 45 and 60 min after the i.c.v. injection. The samples were collected in ice-cooled tubes. Plasma ACTH and β-endorphin concentrations were measured using radioimmunoassay kits for ACTH (Mitsubishi Yuka Co., Tokyo, Japan) and β-endorphin (Peninsula Laboratories, CA).

To determine the effect of a CRH antagonist on plasma ACTH levels after i.c.v. PrRP31 administration, α-helical CRH (2 mg/rat, Peninsula Laboratories, CA) was administered via the indwelling jugular cannula to an active treatment group, while a control group received saline. Fifteen minutes later, both groups received 10 nmol i.c.v. PrRP31. Blood samples were taken 15 and 0 min prior to, and 10, 20, 30, 45 and 60 min after, the administration of α-helical CRH + PrRP31 or saline + PrRP31. All experiments to measure the plasma ACTH concentration were performed between 09:00 and 12:00 h in a day to avoid the influence of circadian rhythm. Plasma ACTH concentrations were measured using the above-mentioned radioimmunoassay kit.

The data were analyzed using a one-way ANOVA with repeated measurements, and differences between treatment groups were evaluated using Dunnett's multiple tests. The statistical significance level was set at $P < 0.05$.

Immunocytochemical staining revealed that i.c.v. administration of PrRP31 (20 nmol/rat) caused marked induction of FOS-RI in the two subdivisions of the PVN, the parvocellular PVN and the magnocellular PVN (Fig. 1B). Double immunostaining for c-Fos and CRH revealed that both FOS-RI- and CRH-positive cell bodies were present in the parvocellular PVN (Fig. 1C,D). About 80% CRH neurons were positive for c-Fos reaction in the PVN.

Double immunostaining for CRH and PrRP was performed with the respective specific antibodies. The results clearly showed contact between some CRH cell bodies and PrRP neurons in the PVN (Fig. 1E,F). The CRH-immunolabeled cell bodies were surrounded by PrRP immunoreactive fibers, suggesting synapse-like contact in this brain region.

Central administration of PrRP31 (10 nmol/rat) caused a significant increase in plasma ACTH levels compared with vehicle-treated control rats. Plasma ACTH levels peaked 15 min after the central injection of PrRP31, then gradually decreased, returning to their initial values after 60 min. No significant increase was observed after the administration of 1 nmol PrRP31. Central administration of PrRP31 (10 nmol/rat) also significantly increased plasma β-endorphin levels (data not shown). In contrast, systemic administration of PrRP31 (50 nmol/kg) did not increase plasma levels of either ACTH or β-endorphin (data not shown).

Intravenous (i.v.) administration of α-helical CRH 15 min before i.c.v. administration of PrRP31 (10 nmol/rat) significantly attenuated ($P < 0.01$) the increase in plasma ACTH levels observed after i.c.v. administration of PrRP31 alone (Fig. 2). The animals that received α-helical CRH had significantly lower ACTH values throughout the 30 min testing period compared with the saline + PrRP31 group. These results indicate that the effects of PrRP31 are dependent on CRH receptor activation.

PrRP was identified as a ligand for hGR3 by a strategy based on reverse pharmacology [3], and may therefore possibly play an important role in the regulation of other biological functions besides PRL secretion. Immunocytochemical studies have demonstrated that PrRP is widespread throughout the brain, but no immunopositive nerve fibers were observed in the external region of the median eminence, where the nerve fibers of the classical hypophysiotropic hypothalamic peptides project [8,17]. These data suggest that PrRP differs from the classical hypothalamic peptides, but may have important functions in the central nervous system other than its PRL releasing activity. Although immunoreactive PrRP is widely distributed in the rat brain [5,8], its function has not yet been discerned. Recently, we demonstrated that PrRP neurons show axosomatic synaptic contact with oxytocin cell bodies located

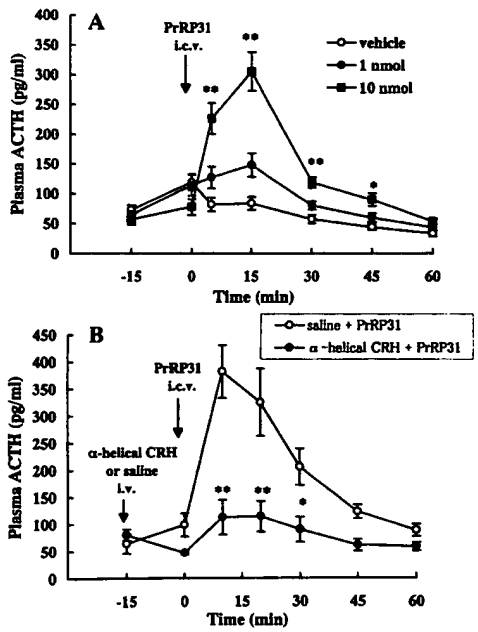

Fig. 2. (A) Effects of intracerebroventricular administration of PrRP31 at doses of 1 nmol/rat (●) or 10 nmol/rat (■), and of the vehicle (PBS containing 0.5% bovine serum albumin) alone (○), on plasma ACTH levels in conscious rats. (B) Effects of pretreatment with α-helical CRH on PrRP31-induced increases in plasma ACTH concentrations. The increase in plasma ACTH after central administration of PrRP31 (10 nmol/rat) was significantly blocked by pretreatment with the CRH antagonist (2 mg/rat). (●) α-helical CRH + PrRP31; (○) saline + PrRP31. Values are means ± SEM ($n = 7$–8). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle alone (A) and saline + PrRP31 (B).

in the PVN or SON [8], and central administration of PrRP31 resulted in increased oxytocin release [9]. These results might reflect the increase in FOS-RI in the magnocellular PVN after central administration of PrRP31 (Fig. 1B). Localization of immunoreactive PrRP has also been reported in the parvocellular neurosecretory cells of the PVN [8] and PrRP receptors have been detected in several hypothalamic nuclei, including the PVN [2,15]. A clue to other possible PrRP functions in the brain was discovered when we found that c-Fos levels in the CRH cell bodies of the parvocellular PVN increased after central administration of PrRP31 (Fig. 1A), and that PrRP neurons made contact with these CRH cell bodies (Fig. 1E,F). These results suggest that PrRP might play a physiological role as a neurotransmitter/neuromodulator in the regulation of CRH neuronal activity.

The present study has provided the first evidence that centrally administered PrRP31 causes a dose-dependent increase in plasma ACTH levels in the rat. The inhibition of PrRP31-induced increases in ACTH by the CRH antagonist, α-helical CRH, indicates that ACTH secretion is induced through CRH. This finding was further supported by morphological studies indicating dramatic FOS-RI accumulation after i.c.v. administration of PrRP31, and showing that PrRP neurons are in direct contact with CRH neurons in the PVN. Thus, we conclude that PrRP is a potent stimulant of CRH neurons in the PVN.

Interestingly, PrRP mRNA-containing cells have been found in two populations of neurons in the brain stem that are catecholaminergic, namely the A1 region in the ventrolateral reticular formation and the A2 region in the nucleus of the solitary tract [1,5,11]. The catecholaminergic neurons originating in the A1 and A2 regions are known to innervate the hypothalamus [13]. In particular, noradrenalin is known to be a central regulator of the activation of hypophysiotropic parvocellular CRH neurons innervated by catecholaminergic terminals [7] and to activate CRH neurons [6]. These results suggest that PrRP may act as a co-transmitter with catecholamines during the activation of hypophysiotropic CRH neurons. However, further studies will be needed to determine whether noradrenalin modifies the effect of PrRP on CRH neurons.

It is noteworthy that particularly strongly immunoreactive PrRP nerve fibers are localized in the BST [8,17]. The lateral BST is reported to have a stress-related function [13] and is known to be innervated by CRH neurons [14]. As PrRP fibers are abundant in the lateral BST, PrRP is likely to act as a regulatory neurotransmitter or neuromodulator on CRH neurons in this area.

In conclusion, during the present study we have revealed that, as well as having PRL-releasing activity, PrRP is able to elicit CRH secretion. This might reflect the anatomical relationship between PrRP fibers and CRH cell bodies in the PVN. Our results suggest that PrRP may have multiple functions in the endocrine and central nervous systems, including a regulatory role in ACTH secretion.

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